Development of a Novel Transgenic Rat Overexpressing the P2Y₂ Nucleotide Receptor Using a Lentiviral Vector

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Key Words

P2Y₂ receptor • Nucleotide • Sjögren's syndrome, transgenic rat

Abstract

The G protein-coupled P2Y₂ nucleotide receptor (P2Y₂R) is upregulated in response to stress and tissue injury and has been postulated to play a role in chronic inflammation seen in atherosclerosis, Alzheimer's disease and Sjögren's syndrome. The role of P2Y₂R upregulation in vivo is poorly understood, in part due to the lack of a P2Y₂R overexpressing animal model. The P2Y₂R overexpressing transgenic rat was generated using a lentiviral vector. Rats overexpressing P2Y₂R showed a significant increase in P2Y₂R mRNA levels in all tissues screened as compared to nontransgenic rats. Fura 2 imaging of smooth muscle cells (SMCs) isolated from aorta indicated that the percentage of cells exhibiting increases in the intracellular free calcium concentration in response to P2Y₂R agonists was significantly greater in freshly isolated SMCs from transgenic rats than wild-type controls. Histopathological examination of tissues revealed that P2Y₂R overexpressing rats develop lymphocytic infiltration in lacrimal glands and kidneys as early as at 3 months of age. These rats show similarities to patients with Sjögren's syn-

drome who display lymphocyte-mediated tissue damage. This transgenic rat model of P2Y₂R overexpression may prove useful for linking P2Y₂R upregulation with chronic inflammatory diseases, neurodegenerative diseases and Sjögren's syndrome.

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Introduction

Extracellular nucleotides bind to cell surface receptors known as P2 receptors that are present in most tissues [1]. These receptors have been classified into two main families: the P2X receptors that are ligand-gated ion channels comprised of homo- or heterodimers [2], and P2Y receptors that are seven membrane spanning receptors coupled via G proteins to phospholipase C and/or adenylate cyclase [3]. The G protein-coupled P2Y₂ receptor (P2Y₂R) is equipotently activated by ATP or UTP and is believed to be involved in the regulation of proliferation and differentiation of target cells [3]. Activation of the P2Y₂R causes proliferation and/or migration of human epidermal keratinocytes, lung epithelial tumor cells, glioma cells, smooth muscle cells (SMCs), endothelial cells, and primary rat astrocytes [reviewed in 4]. P2Y₂R activation

also increases neuronal differentiation and growth and inhibits apoptosis in neurons [5, 6]. In addition, the P2Y₂R interacts with integrins and growth factor receptors to activate multiple signaling pathways and regulate reactive astrogliosis associated with neurodegenerative diseases [7]. P2Y₂R activation induces α -secretase-dependent cleavage of amyloid precursor protein (APP) to release the nonamyloidogenic peptide, sAPP α , suggesting a neuroprotective role for the P2Y₂R [8]. Furthermore, activation of the P2Y₂R increases hepatocyte resistance to hypoxia [9]. Chen et al. [10] reported the involvement of the P2Y₂R in neutrophil chemotaxis in which activation of the P2Y₂R by ATP enables signal amplification and controls gradient sensing and migration of neutrophils.

The P2Y₂R is upregulated in response to tissue injury or stress [11]. P2Y₂R activation accelerates the rate of corneal epithelial healing [12] and plays a role in the wound healing process in rat epidermis [13]. P2Y₂R mRNA levels and/or activity increase in models of salivary gland stress or disease [14, 15] and in blood vessels after balloon angioplasty [16] or stress induced by insertion of a vascular collar [17]. Upregulation and activation of P2Y₂Rs in endothelium induces development of intimal hyperplasia associated with atherosclerosis and restenosis [17], although activation of endothelial cell P2Y₂Rs expressed at endogenous levels promotes vasodilatation [18–20]. In addition, P2Y₂R activation inhibits bone formation by osteoblasts [21] consistent with the 9–17% increase in bone mineral content of P2Y₂R knockout mice as compared to wild-type (WT) mice [22]. Studies also have indicated that extracellular nucleotides induce P2Y₂R-mediated secretion of interleukin-6 by airway epithelia [23]. The P2Y₂R knockout mouse exhibits significantly lower levels of IL-6, suggesting a protective role for the P2Y₂R against lung infections [24]. P2Y₂R polymorphism also has been linked to cystic fibrosis and P2Y₂R activation mediates an increase in Cl[−] secretion and inhibition of Na⁺ absorption in airway epithelial cells [25] that is virtually absent in the trachea of P2Y₂R knockout mice [26].

Transgenic (Tg) animals are widely used in life science research to study the role of gene expression in vivo. Although mice have been used extensively in many areas of biomedical research, Tg rats have certain advantages over mice due to their larger size, unique genetics, and well-studied behavioral characteristics [27]. Rats are also better suited for microsurgery, cell and tissue transplantation, in vivo functional analyses and studies that require multiple sampling [28]. Recently, germ line transmission and ubiquitous expression of a transgene has been demonstrated in Tg rats created using a lentiviral vector [29,

30]. It has been reported that lentiviral vector injection into the perivitelline space of zygotes yields a much higher Tg rate compared to the conventional means of injecting plasmid DNA constructs into the pronucleus of a zygote [31].

In the present study, we describe the generation of a novel Tg rat line that overexpresses the P2Y₂R using a lentiviral vector. To date, there is no report of production of a P2Y₂R overexpressing animal model. The Tg rat overexpressing the P2Y₂R will be a very useful tool to investigate the consequence of P2Y₂R upregulation in vascular inflammation, neurodegenerative diseases and the autoimmune disorder Sjögren's syndrome where the P2Y₂R has been suggested to play a pathophysiological role.

Methods

Lentiviral Vector Production and Titration

Rat total RNA was reverse transcribed using oligo-dT primer. The product of this reaction was amplified using a forward primer (5'-GTATTCGGATCCTGCGAGTGAAGAACTGGAA-CGGA-3') that anneals 97 bp upstream of the translation start site and a reverse primer (5'-ATGCAGAATTCTTTTTTTT-TTTTTTTTTT-3') that anneals to the poly-A tail. The forward primer contains a *Bam*HI site (underlined sequence) and the reverse primer contains an *Eco*RI site (underlined sequence) to generate flanking regions for ligation. Amplified P2Y₂R cDNA was ligated into the pLV-EGFP vector (obtained from Dr. C. Lois, MIT, Cambridge, Mass., USA) where the P2Y₂R cDNA replaced the enhanced green fluorescence protein sequence [29]. The new vector, designated as pLV-P2Y₂R, was sequenced to confirm the validity of the polymerase chain reaction (PCR) amplification and ligation. The expression of the P2Y₂R in pLV-P2Y₂R-infected cells was driven by the ubiquitin-C (UB) promoter that drives expression of ubiquitous transgenes [32]. The pLV-P2Y₂R is composed of the woodchuck hepatitis virus posttranscriptional regulatory element (WRE) and an HIV-flap element at the 5'-LTR (fig. 1). Lentivirus was generated by cotransfection of pLV-P2Y₂R, p Δ 8.9 (composed of structural genes for virion assembly) and pVSV-G (Invitrogen, Carlsbad, Calif., USA) into a 293FT packaging cell (Invitrogen). Culture medium was collected at 48 h after transfection for 3 consecutive days at 24-hour intervals. The supernatant was centrifuged at 25,000 g for 90 min. The viral vector pellet was resuspended in Hanks' balanced salt solution, aliquoted, titered and kept frozen at −80°C. Direct measurements of the titer of pLV-P2Y₂R were not possible due to the lack of an anti-P2Y₂R-specific antibody. Therefore, the titer of pLV-P2Y₂R was estimated to be in the range of 1 × 10⁸ to 1 × 10⁹ CFU/ml based on a similar study using the same transfection conditions with a lentiviral vector expressing the enhanced green fluorescence protein (EGFP) [31].

Embryo Production and Microinjection

Sprague-Dawley (SD) female rats (28–30 days old) were purchased from Harlan Sprague Dawley (Indianapolis, Ind., USA) and superovulated using subcutaneous implantation of 8 units of

Fig. 1. DNA construct of pLV-P2Y₂R. LTR = Long terminal repeat; HIV-1-FLAP = human immunodeficiency virus-1 flap element; UB = ubiquitin-C promoter; WRE = woodchuck hepatitis virus posttranscriptional regulatory element.

follicle-stimulating hormone, introduced via Alzet mini-osmotic pumps and followed by an intraperitoneal injection of 15 units of luteinizing hormone (LH) approximately 50–52 h after follicle-stimulating hormone implantation [33]. To obtain zygotes, the donor rats were mated with SD male rats just after LH injection. Donor rats were sacrificed and the oviducts were removed to collect zygotes in HEPES-buffered Tyrode's lactate solution [34] approximately 20–24 h after LH injection. Morphologically normal embryos having both male and female pronuclei and sperm tail were used for lentiviral vector injection. For microinjection, a sharp, pointed pipette was transferred into a BL-II hood for vector loading. The vector was then loaded into the injection pipette using a microloader. Injections were done in a 100- μ l drop of HEPES-buffered Tyrode's lactate covered with mineral oil. Each zygote was immobilized with a holding pipette and then injected with the viral vector into the perivitelline space.

Embryo Transfer

Embryo transfers were done surgically [35]. Lentiviral vector-injected zygotes were transferred into an 8- to 10-week-old pseudopregnant SD recipient rat. The recipient rats were synchronized by intraperitoneal injection with 40 μ g of gonadotropin-releasing hormone analog des-Gly10 [D-Ala6] ethylamide (Sigma). Pseudopregnancy was verified by the presence of a vaginal plug. Injected embryos were loaded into a fine glass pipette, the pipette was then inserted into the infundibulum, and embryos were discharged into the oviduct of the recipient.

Animals and Breeding

SD outbred rats were used to generate Tg rat strains. One PCR-positive P2Y₂R Tg founder was mated with a WT SD rat to determine germline transgenesis. The P2Y₂R overexpressing rat strain was bred for 6 generations to ensure stability of the transgene. All animal studies were performed in accordance with the University of Missouri's Animal Care and Use Committee guidelines and the ILAR Guide for the Care and Use of Laboratory Animals.

Genomic DNA Isolation and PCR

Genomic DNA from tail snip samples was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, Wisc., USA), according to the manufacturer's instructions. PCR was used for screening of Tg animals. Primers annealing at 1,205–1,226 bp of the UB promoter (5'-GTCCGCTAAATTCTGGCCGTT-3') and 431–451 bp of the P2Y₂R transgene (5'-ACTGTGC-TAAATGGCCAGTGGT-3') were used in PCRs to yield a 499-bp amplification product. The 50- μ l reactions were carried out using 50 ng of genomic DNA, 100 ng of each primer and 0.5 U of Biolase Taq (Bioline, Randolph, Mass., USA). The PCR products were size separated through a 1% (w/v) agarose gel and stained with ethidium bromide for visualization.

Northern Blot Analysis

Total RNA was isolated from P2Y₂R Tg (n = 5) and WT (n = 5) rats using Trisure (Bioline). Aorta, brain, heart, kidney, lacrimal gland, liver, lung, muscle and salivary gland total RNAs were used in Northern blot analysis. The total RNAs were size separated through 1% (w/v) agarose gels before transferring to Genescreen Plus membranes (Perkin Elmer, Waltham, Mass., USA) overnight. The 529-bp P2Y₂R probe template was prepared by amplification of pLV-P2Y₂R using forward (5'-ACACCCTCAACGC-CATCAACAT-3') and reverse (5'-AATGGCAGCTGTTTGCAT-GGGA-3') primers annealing at 930–951 bp and 1,437–1,458 bp of the P2Y₂R cDNA in pLV-P2Y₂R, respectively. The ³²P-labeled probe was generated using the probe template, Ready-To-Go DNA Labeling Beads (Amersham Biosciences, Piscataway, N.J., USA) and [α -³²P]-dCTP (3,000 Ci/mmol specific activity; Perkin Elmer, Wellesley, Mass., USA). The membranes were prehybridized in 10% (w/v) dextran sulfate, 5 $\times$ SSPE (0.75 M NaCl, 50 mM NaH₂PO₄·H₂O, 5 mM EDTA), 50% (v/v) formamide, 5 $\times$ Denhardt's reagent, and 1% (w/v) SDS at 42°C for 6 h prior to hybridization with the P2Y₂R probe overnight followed by exposure to BioMax MS autoradiography film. Membranes were rehybridized with 18S rRNA probe to correct for pipetting differences. The intensity of bands was determined using Kodak D v 3.6.3 software (New Haven, Conn., USA).

Isolation of Rat SMCs

Rat aortic SMCs were obtained from the 4- to 6-week-old rats. Thoracic aorta was denuded of adventitia, cut into 1-mm cubes and digested in 0.1% (w/v) collagenase in Dulbecco's modified Eagle's medium containing 1% (w/v) bovine serum albumin at 37°C with gentle rocking. Cells dissociated within the first 30 min were discarded to improve SMC purity. Liberated cells were resuspended in Dulbecco's modified Eagle's medium with penicillin (50 IU ml⁻¹), streptomycin (50 μ g ml⁻¹) and L-glutamine (2 mM). These cells were 100% positive for smooth muscle actin immunofluorescence.

Intracellular Free Ca²⁺ Concentration Measurements

The intracellular free calcium concentration, [Ca²⁺]_i, in single SMCs extracted from the aorta was determined using fura 2, a Ca²⁺-sensitive fluorescent dye, and an InCyt Dual-Wavelength Fluorescence Imaging System (Intracellular Imaging, Cincinnati, Ohio, USA). Briefly, cells were plated on coverslips and incubated at 37°C for ~6 h to allow adherence. Adherent cells were incubated with 2.0 μ M fura 2-acetoxymethyl ester in assay buffer for 40 min at 37°C in the dark. Cells were washed and staged under the fluorescence microscope, stimulated with agonists at 37°C and exposed to 340/380 nm light. Fluorescence emission at 505 nm was converted to [Ca²⁺]_i using a standard curve constructed with solutions containing known calcium concentrations. Ago-

Table 1. Tg efficiency after injection of lentiviral vectors carrying P2Y₂R under the control of the UB promoter in SD rat zygotes

Zygotes injected	Embryos transferred	Pups born	Germline Tg
22	20 (91%)	12 (60%)	1 (8%)

nist-induced increases in $[Ca^{2+}]_i$ were calculated by subtracting basal $[Ca^{2+}]_i$ from the peak $[Ca^{2+}]_i$.

Histological Examination

Brain, exorbital lacrimal gland, heart, intestines, kidney, liver, lung, lymph nodes, pancreas, salivary glands, skin and spleen were collected from 3-, 5-, 6-, 8- and 12-month-old P2Y₂R overexpressing rats as well as from WT controls. Tissues were fixed in 4% (v/v) paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Histopathology of the tissues was analyzed under an upright microscope (Zeiss Axiophot, Germany). Kidney lesions were visualized with Congo red to detect amyloid, Picrosirius red (PSR) to detect collagen and periodic acid methenamine (PAM) to detect basement membranes. Adobe Photoshop CS3 (Adobe, San Jose, Calif., USA) was used to convert digital PSR images to grayscale and collagenous material was depicted at relative grayscale values above 127. Images were analyzed using Image J software (NIH, Bethesda, Md., USA) to quantify the percentage of pixels in the image that stained PSR positive for collagen.

Clinical Blood Chemistry and Hematology

Blood samples from two P2Y₂R overexpressing rats (5 and 8 months old) and a WT rat were analyzed for urea nitrogen, creatinine, total protein, albumin, globulin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), uric acid, chloride, calcium and phosphorus levels. In addition, samples were analyzed for red blood cells, hemoglobin, hematocrit and white blood cells at the University of Missouri Research Animal Diagnostic Laboratory (<http://www.radil.missouri.edu>).

Serological Testing

Rat colonies were monitored every 3 months by the University of Missouri Research Animal Diagnostic Laboratory for the absence of the following pathogens: *Mycoplasma pulmonis*, rat parvovirus (NS-1), Toolan's H-1 virus, Kilham rat virus, rat minute virus, pneumonia virus of mice, rat coronavirus, sialodacryoadenitis virus, Theiler's meningoencephalitis virus and rat theilovirus.

Statistical Analysis

To determine statistically significant differences in gene expression and $[Ca^{2+}]_i$ between P2Y₂R overexpressing Tg and WT rats, general linear models of SAS version 9.1 (Cary, N.C., USA) were used. The values are given as the mean $\pm$ standard error of the mean (SEM). For all statistical tests, the level of significance was chosen as $p < 0.05$.

Results

Tg Efficiency, Initial Screening of a P2Y₂R Tg Founder and Generation of Homozygous Tg Animals

One SD recipient was used to carry embryos injected with pLV-P2Y₂R. Out of 22 embryos, one PCR-positive P2Y₂R overexpressing Tg rat was generated yielding a Tg generation rate of $\sim 5\%$ (table 1). The P2Y₂R Tg rats were bred for 6 generations.

P2Y₂R mRNA Expression in Tg and WT Rats

Expression of P2Y₂R mRNA was determined by Northern blot analysis (fig. 2) in 6-week-old rats at the third generation ($n = 2$). P2Y₂R mRNA was highly expressed in all tissues screened in Tg rats (fig. 2a). There was a dramatic 58-fold ($p < 0.05$) increase in P2Y₂R mRNA expression in brain tissue of Tg rats as compared to brain tissue from WT rats that barely expressed P2Y₂R mRNA. P2Y₂R mRNA levels in Tg rats were also significantly increased in heart (8-fold; $p < 0.05$), kidney (33-fold; $p < 0.05$), liver (2.8-fold; $p < 0.05$), lung (2.5-fold; $p < 0.05$), and leg muscle (2.8-fold; $p < 0.05$) as compared to tissues from age-matched WT control rats (fig. 2b).

To confirm the stability of transgene expression, P2Y₂R mRNA levels were analyzed in tissues of three Tg rats from the sixth generation. P2Y₂R mRNA levels remained elevated in all Tg tissue specimens examined (fig. 2c, d), consistent with results obtained with third generation Tg rats (fig. 2b). These results showed that sixth generation P2Y₂R overexpressing Tg rats ($n = 3$) had 106-, 74-, 55-, 1.9-, 2.3- and 25-fold higher P2Y₂R mRNA expression in brain, kidney, lacrimal gland, liver, lung and salivary gland, respectively, as compared to tissues from WT control rats. P2Y₂R mRNA expression in brain and salivary and lacrimal glands was not detectable in WT animals (fig. 2c, d). Since P2Y₂R mRNA levels in brain and salivary and lacrimal glands could not be determined in WT animals, expression levels of P2Y₂R mRNA in these tissues were assumed to be 50% of the levels in WT kidney for the purpose of quantitative comparisons with tissues from P2Y₂R overexpressing rats. P2Y₂R mRNA levels were also found to be significantly higher in Tg rat aortic SMCs as compared to WT rat aortic SMCs (fig. 3a).

Functional Expression of the P2Y₂R Transgene in Aortic SMCs

To determine whether the P2Y₂R transgene was functional, freshly isolated aortic SMCs from WT and P2Y₂R overexpressing Tg rats (fig. 3a) were exposed to UTP or

Fig. 2. Gene expression analysis of P2Y₂R mRNA levels in Tg and WT rats. **a** Northern blot hybridization of total RNA from tissues of P2Y₂R Tg and WT rats. P2Y₂R expression in tissue specimens from 2 third-generation Tg rats was compared to 2 WT rats. Total RNA was hybridized with a P2Y₂R probe (upper band in autoradiogram) prior to hybridization with 18S rRNA (lower band in autoradiogram). **b** P2Y₂R mRNA expression in organs obtained from third-generation P2Y₂R overexpressing Tg (n = 2) and WT (n = 2) rats. The expression of P2Y₂R mRNA was normalized to 18S rRNA. **c** Northern blot hybridization of total RNA from tissues of sixth-generation P2Y₂R overexpressing Tg (n = 3) and WT (n = 3) rats. Total RNA was hybridized with a P2Y₂R probe (upper band in autoradiogram) prior to hybridization with 18S rRNA (lower band in autoradiogram). **d** P2Y₂R mRNA expression in organs obtained from 3 sixth-generation P2Y₂R overexpressing Tg and WT rats. The expression of P2Y₂R mRNA was normalized to 18S rRNA. B = Brain; H = heart; K = kidney; LG = lacrimal gland; Li = liver; Lu = lung; M = muscle (leg); S = salivary gland. * p < 0.05 statistically significant differences in expression levels of P2Y₂R mRNA between tissues from P2Y₂R overexpressing Tg and WT animals.

Fig. 3. P2Y₂R mRNA expression and receptor activity. **a** P2Y₂R mRNA expression in aorta from WT and fourth-generation P2Y₂R overexpressing Tg rats relative to 18S rRNA. **b** [Ca²⁺]_i measurements in single SMCs isolated from aortas of WT or fourth-generation P2Y₂R Tg rats. Increases in [Ca²⁺]_i were expressed by subtracting the basal [Ca²⁺]_i (prior to addition of 10 μM UTP or ATP) from the peak agonist-induced increase in [Ca²⁺]_i. Data represent the means ± SEM of results from three independent experiments. * p < 0.05 indicates a significant difference from WT control.

ATP and the [Ca²⁺]_i was measured (fig. 3b). Percentages of aortic SMCs from WT rats that responded to agonist were 36% (25/69) for ATP and 23% (18/76) for UTP. In contrast, 90% (49/54) and 94% (61/65) of SMCs isolated

from P2Y₂R overexpressing Tg rats responded to UTP and ATP, respectively. P2Y₂R agonist-induced intracellular calcium mobilization was greater in P2Y₂R overexpressing Tg rats as compared to WT rats (fig. 3b).

Fig. 4. Histopathological images of lacrimal gland, kidney, and liver from P2Y₂R overexpressing and WT rats. Representative histopathological images of exorbital lacrimal gland from 3-month-old (**a**) and 8-month-old (**b**) rats; kidney from 3-month-old (**d**) and 1-year-old (**e**) rats and liver from 5-month-old (**g**) and 1-year-old (**h**) P2Y₂R overexpressing rats and 6-month-old WT rat (**c**, **f**, **i**). The tissues were fixed in 4% (v/v) paraformaldehyde and stained with hematoxylin and eosin. **a** A large area of perivascular and periductal lymphocyte infiltration (black arrows) in a 3-month-old P2Y₂R overexpressing rat. **b** Harderian gland alteration of the exorbital lacrimal gland in a P2Y₂R overexpressing rat (white arrows); interstitial and periductal lymphocyte infiltrates (black arrows). **d** Glomerulonephropathy in a P2Y₂R overexpressing rat with glomerular sclerosis (white arrow), dilatation of tu-

bular lumen and tubular proteinaceous casts (arrowheads), interstitial lymphocyte infiltrates (black arrows). **e** Glomerulonephropathy in a P2Y₂R overexpressing rat showing thickened glomerular basement membranes and glomerular adhesion (white arrows), thickened tubular basement membranes with tubular regeneration (black arrowheads, blue in the online version), interstitial lymphocyte infiltrates (black arrows) and a small proteinaceous cast (white arrowhead, yellow in the online version). **g**, **h** Representative images of mild pericholangial and perivascular lymphocytic infiltrates (black arrows) and scattered mast cells (arrowheads) in portal triads of P2Y₂R overexpressing rats. **c**, **f**, **i** WT exorbital lacrimal gland, kidney, and liver, respectively. The yellow scale bars represent a distance of 200 μ m for **a**, **c**, **d**, **f**, **g**, and **i** and 100 μ m for **b**, **e**, and **h**.

Histological Examination

Lesions were identified in multiple tissues from P2Y₂R overexpressing Tg rats with the most remarkable changes occurring in the exorbital lacrimal gland (fig. 4a, b) and kidney (fig. 4d, e). Lymphocytic infiltrates were also ob-

served in the exorbital lacrimal gland, kidney and liver of P2Y₂R overexpressing Tg rats but were absent in the WT rat (fig. 4).

The exorbital lacrimal glands of P2Y₂R overexpressing Tg rats displayed marked multifocal harderian gland al-

Fig. 5. Histological examination of kidney sections from P2Y₂R overexpressing Tg (a, c) and WT (b, d) rats in which basement membranes were stained with PAM (a, b) and collagen was stained with PSR (c, d). Basement membrane thickening in affected glomeruli, Bowman's capsules, and tubules is indicated by black arrows, whereas PSR-positive collagen in affected glomeruli and

Bowman's capsules is indicated by white arrows. Analysis of pixels in the images using Image J software indicated that 19.3% of pixels in image C stained PSR-positive for collagen, while 3.6% of kidney cells stained positive for PSR in P2Y₂R overexpressing and WT rats, respectively. The scale bars represent a distance of 200 μ m.

terations, characterized by acinar atrophy and replacement of normal alveolar cells with distinct lumen by cuboidal to columnar cells with finely vacuolated, eosinophilic cytoplasm (fig. 4a, b). Moderate multifocal perivascular and periductular lymphocytic infiltrates were present within lacrimal glands of P2Y₂R overexpressing Tg rats, with lymphocytic infiltrates occasionally extending into the glandular interstitium (fig. 4a, b). Scattered mast cells were commonly observed in interlobular connective tissue and diffusely within the interstitium (fig. 4b). These lesions were absent in the WT rat (fig. 4c).

Glomerulonephropathy, ranging from mild to severe, was identified in the kidneys of P2Y₂R overexpressing Tg rats. Mild nephropathy in 2 Tg rats, aged 5 and 6 months, was characterized by multifocal basophilia and hyperplasia in proximal tubules, renal tubular dilation and proteinaceous cast deposition. Mild multifocal mineralization was also observed within the tubular lumen (not shown). Moderate glomerulonephropathy, observed in an 8-month-old P2Y₂R overexpressing Tg rat, was characterized by mild multifocal glomerulosclerosis, glomerular adhesions and parietal cell hyperplasia (not shown), moderate multifocal dilation of tubular lumina with fre-

Table 2. Clinical blood chemistry and hematology of P2Y₂R overexpressing Tg and WT rats

Clinical blood chemistry	P2Y ₂ R 1	P2Y ₂ R 2	WT 1
Urea nitrogen, mg/dl	19	21	24
Creatinine, mg/dl	0.4	0.3	0.3
Uric acid, mg/dl	5.8	1.5	1.9
Total protein, g/dl	7.3	7.4	7.3
Albumin, g/dl	3.5	3.3	3.6
Globulin, g/dl	3.8	4.1	3.7
ALT, U/dl	49	72	29
ALP, U/dl	279	258	125
Calcium, mg/dl	12.2	11.3	11.9
Phosphorus, mg/dl	9.1	7.8	10.5
Chloride, mmol/dl	101	98	99
Hematology			
WBC, × 10 ³ /μl	15.1	10.2	8.6
Segmented neutrophils, × 10 ³ /μl	1.06	0.92	0.35
Band neutrophils, × 10 ³ /μl	0	0	0
Lymphocytes, × 10 ³ /μl	13.9	9.3	8.2
Monocytes, × 10 ³ /μl	0	0	0
Eosinophils, × 10 ³ /μl	0	0	0
Basophils, × 10 ³ /μl	0	0	0
Red blood cell parameters			
RBC, × 10 ⁶ /μl	9.23	8.21	7.92
Hemoglobin, g/dl	15.8	15.1	15.0
Hematocrit, %	45.9	44.3	42.8
MCV, fl	49.7	53.9	54.0
MCH, pg	17.1	18.3	19.0
MCHC, g/dl	34.4	34.0	35.2

Values in bold indicate differences greater than 25% between P2Y₂R overexpressing Tg rats and WT rats. Rats P2Y₂R 1 and P2Y₂R 2 were 5 and 8 months old, respectively, and WT 1 was 6 months old.

quent proteinaceous tubular casts, and occasional mineral deposition within tubular lumina. Mild multifocal perivascular and interstitial lymphocytic infiltrates (fig. 4d, e) were also observed. Marked multifocal glomerulonephropathy was observed in 2 P2Y₂R overexpressing Tg rats, aged 3 and 12 months (fig. 4d, e), with increased severity and frequency of lesions described in the 8-month-old Tg rat, as well as marked multifocal tubular regeneration, thickening of glomerulotubular basement membranes, and moderate interstitial fibrosis (fig. 4d, e). These lesions were absent in the WT rat (fig. 4f).

Tg rats displayed mild, but increased pericholangial and perivascular lymphocyte infiltration in portal triads as compared to the WT rat. Occasional scattered mast cells were observed within Tg portal triads but were ab-

sent in the WT rat (fig. 4g, h). No pathological lesions were observed in brain, heart, intestines, lymph nodes, pancreas, salivary gland, skin or spleen of P2Y₂R overexpressing Tg or WT rats (not shown).

PAM staining of kidney tissue from a P2Y₂R overexpressing rat showed basement membrane thickening in glomerular lesions, Bowman's capsules and affected tubules (fig. 5a) as compared to kidney tissue from a WT rat (fig. 5b). Basement membrane thickening was attributed to increased collagen accumulation as evidenced by increased PSR staining of kidney tissue from a P2Y₂R overexpressing rat (fig. 5c) as compared to WT controls (fig. 5d). Image J analysis revealed that 19.3 and 3.6% of P2Y₂R overexpressing and WT rat kidney cells, respectively, stained positive for collagen. All tissue sections were negative for Congo red stain, indicating the absence of amyloid deposition (not shown).

Clinical Blood Chemistry and Hematology

The majority of blood parameters were similar between P2Y₂R overexpressing Tg and WT rats (table 2). However, ALT and ALP were elevated in both Tg animals as compared to the WT rat, and uric acid was comparatively elevated in the 5-month-old Tg rat, which suggested damage to liver and kidney, respectively. Absolute lymphocyte count was 60% greater in P2Y₂R overexpressing Tg rats as compared to the WT rat. P2Y₂R overexpressing rats also had comparatively elevated absolute counts of segmented neutrophils and lymphocytes (table 2).

Discussion

Tg animal technology has led to significant advances in our understanding of the pathology of human diseases and thus provides an ideal experimental system to study gene function in vivo [36]. Although Tg mice are the most commonly used animal models in the biomedical field due to the high efficiency of their production, development of efficient gene transfer technologies in the rat is also critical for the broader application of genetically modified animal models to the investigation of molecular mechanisms underlying human diseases [37, 38]. Unfortunately, there are few Tg rat lines available as compared to thousands of Tg mouse lines. To date, the pronuclear injection (PI) method has been used to generate the majority of Tg rats. Generating Tg rats using PI is difficult due to technical and physiological limitations, such as high postinjection cell death (35–70%) and low transgene integration rate [27, 39, 40].

A relatively new and efficient means of gene transfer (i.e., using a lentiviral vector) enabled us to produce a Tg rat line overexpressing the P2Y₂R. Oncoretroviral vectors are considered to be a most efficient gene delivery system but are not without their problems, including size limitation (10 kb), multiple integration sites per genome, and difficulties with maintaining transgene expression over subsequent generations [41, 42]. However, recent studies showed that, unlike traditional oncoretroviral vectors, transgenes introduced by lentiviral vectors into oocytes, male germ-line cells, embryos, and embryonic stem cells remain stably expressed [29, 43, 44]. Furthermore, since the lentiviral vector is simply deposited into the perivitelline space of a zygote, there is no need for visualization of the pronuclei, making Tg animal production much easier and less detrimental. Lentiviral vector injection into the perivitelline space yields a much higher Tg rate as compared to the highly inefficient pronuclear microinjection of plasmids in the rat. To generate an average of 4–6 Tg founder rats with PI, for example, it is necessary to inject 500–600 zygotes if the background strain is outbred (such as for SD rats) and to prepare about 20 recipient females for embryo transfer. In addition to the large numbers of microinjections and embryo transfers with the PI method, it is also necessary to genotype and maintain a large number of pups to identify Tg rats. These shortcomings are magnified if one wishes to create a Tg rat on an inbred background, such as Fischer 344, Lewis, Brown Norway or Wistar-Furth, due to their poor response to superovulation and higher sensitivity to microinjection [27, 45]. This study demonstrates that injection of only 22 SD zygotes obtained from one female donor and transferred to one recipient was sufficient to create one founder that was germline Tg, and transgene expression was maintained through at least 6 generations. Therefore, this result highlights the effectiveness of generating Tg rats using lentiviral vectors.

Although the P2Y₂R has been studied in a wide variety of cell and tissue types, Tg animal models overexpressing the P2Y₂R were not available before this study. Here, a Tg rat model overexpressing the P2Y₂R was developed that expresses increased levels of P2Y₂R mRNA in major organs and tissues, such as aorta, brain, heart, kidney, liver, lung, lacrimal gland and leg muscle. Comparison of WT and P2Y₂R overexpressing Tg rats showed that expression levels of the P2Y₂R transgene in tissues did not change between the third and sixth generations. The endogenous P2Y₂R in WT rats is expressed in liver, lung and muscle and to a lesser extent in heart, kidney, and brain, but was undetectable in lacrimal and salivary glands. Expression

of the P2Y₂R transgene in lacrimal and salivary glands was elevated as compared to WT rats, but was lower than in the other tissues analyzed. Although oncoretroviral vectors have been considered as efficient gene delivery systems, previous studies showed their limitation with regard to transgene transcriptional silencing in subsequent generations [41]. In this study, we demonstrated that incorporation of the P2Y₂R transgene into a lentiviral vector enabled stable P2Y₂R mRNA expression through 6 generations and the expressed P2Y₂R was functionally active, as indicated by increased P2Y₂R agonist-induced intracellular calcium mobilization in single SMCs from P2Y₂R overexpressing Tg versus WT rats. In addition, there was a significant increase in the percentage of responsive aortic SMCs of P2Y₂R overexpressing Tg versus WT rats. Overall, these results indicate that functional P2Y₂R expression is significantly higher in Tg rat tissue as compared to WT controls.

In the cardiovascular system, it has been shown that extracellular UTP or ATP induces mitogenic activation of SMCs through activation of P2Y₂Rs. P2Y₂R mRNA is upregulated in intimal lesions of rat aorta and contributes to the development of intimal hyperplasia [17]. The placement of a silicone collar around a rabbit carotid artery induced intimal thickening that was preceded by the upregulation of P2Y₂R mRNA in medial SMCs and endothelial cells. In the P2Y₂R overexpressing rat developed in the current study, P2Y₂R-mediated increases in $[Ca^{2+}]_i$ in aortic SMCs were 1.8 times greater than in WT rat aortic SMCs (fig. 3b). Similarly, P2Y₂R mRNA expression in aorta was 2.7 times greater in P2Y₂R overexpressing Tg rats as compared to WT rats (fig. 3a), indicating a close correlation between increased P2Y₂R mRNA expression and functional activity and suggesting that these Tg rats will be ideal for investigations on the role of P2Y₂R overexpression in vascular lesion development.

In this study, we observed pathological changes in lacrimal glands from P2Y₂R overexpressing rats that resemble Sjögren's syndrome, such as lymphocytic infiltration of the lacrimal gland (fig. 4). Masaki and Sugai [46] reported that 45% of patients with Sjögren's syndrome develop symptoms only in exocrine glands, whereas 50% of Sjögren's syndrome patients exhibit lymphocyte-mediated tissue damage that may involve the pulmonary, renal, hepatic, hematologic, and/or dermatologic systems. There are several case reports linking Sjögren's syndrome and renal pathology [47, 48] that resembles the renal histopathology of P2Y₂R overexpressing Tg rats (fig. 4, 5). The glomerulonephropathy observed was associated with basement membrane thickening and collagen depo-

sition, but not amyloid deposition, as determined by PSR, PAM and Congo red staining, respectively. While both harderian gland alteration and chronic progressive nephropathy are known to occur spontaneously in laboratory rats [49], it is notable that all P2Y₂R overexpressing Tg rats exhibited lesions of the lacrimal gland or kidneys regardless of age, whereas no lesions were apparent in lacrimal glands or kidneys of WT rats. Additionally, the severity of lesions, especially involving glomeruli, in 3-month-old P2Y₂R overexpressing Tg rat is incongruent with spontaneous chronic progressive nephropathy. Since the P2Y₂R overexpressing Tg rats are maintained in facilities free of major rat pathogens, as determined by sentinel monitoring, it is unlikely that the lesions in P2Y₂R overexpressing Tg rats are attributable to infectious disease. Pathological lesions observed in P2Y₂R overexpressing animals worsened with age except in the case of the 3-month-old rat which displayed severe lesions in kidney and lacrimal gland. We did not determine P2Y₂R expression levels in animals that were used for histopathological examination. It is possible that the 3-month-old rat expressed higher P2Y₂R levels as compared to other rats used for histopathological examination, since this rat was an offspring of two P2Y₂R Tg parents and may have been homozygous. The other rats used in histopathological examination were confirmed to be heterozygous. Also, since SD rats are outbred, greater individual variations are expected.

Clinical pathology revealed increased levels of ALP, ALT, and uric acid in the blood of P2Y₂R overexpressing Tg rats as compared to WT controls, which was suggestive of kidney and liver abnormalities (table 2). P2Y₂R overexpressing rats also had comparatively elevated absolute counts of segmented neutrophils and lymphocytes (table 2), which is suggestive of an ongoing inflammatory process. The results of the clinical pathology analysis were confirmed by histopathology and suggest that clinical pathology may be a useful way to monitor the pro-

gression of phenotypic manifestations in P2Y₂R overexpressing Tg rats.

Although P2Y₂R overexpressing Tg rats displayed lymphocytic infiltrates of the kidney and lacrimal gland, a common histopathological marker of Sjögren's syndrome [50], we did not detect pathological changes in salivary gland tissue from P2Y₂R overexpressing Tg rats, consistent with a low level of P2Y₂R mRNA expression in salivary gland (fig. 2) that is perhaps due to tissue-specific mRNA degradation or low transcriptional activity of the UB promoter. Finally, the increase in P2Y₂R activity in SMCs from P2Y₂R overexpressing Tg rats, as compared to WT rats (fig. 3), is consistent with intimal thickening of arteries [16, 17] that also has been reported in female patients with Sjögren's syndrome [51].

Conclusions

P2Y₂R activity has been associated with human disease pathologies in cystic fibrosis [52], Sjögren's syndrome [53] and atherosclerosis [17]. The P2Y₂R overexpressing Tg rat generated for this study may be a useful animal model for investigating the effects of P2Y₂R up-regulation on the development of inflammatory and autoimmune diseases. In addition, since P2Y₂R expression is increased in multiple tissues from P2Y₂R overexpressing Tg rats as compared to WT controls, studies with this Tg rat line may provide novel insights into the roles of the P2Y₂R under various physiological and pathophysiological conditions.

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References

- Boeynaems JM, Communi D, Gonzalez NS, Robaye B: Overview of the P2 receptors. *Semin Thromb Hemost* 2005;31:139–149.
- Khakh BS, North RA: P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 2006;442:527–532.
- Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA: International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006;58:281–341.
- Erb L, Liao Z, Seye CI, Weisman GA: P2 receptors: intracellular signaling. *Pflugers Arch* 2006;452:552–562.
- Arthur DB, Akassoglou K, Insel PA: P2Y₂ receptor activates nerve growth factor/TrkA signaling to enhance neuronal differentiation. *Proc Natl Acad Sci USA* 2005;102:19138–19143.

- 6 Arthur DB, Georgi S, Akassoglou K, Insel PA: Inhibition of apoptosis by P2Y₂ receptor activation: novel pathways for neuronal survival. *J Neurosci* 2006;26:3798–3804.
- 7 Weisman GA, Wang M, Kong Q, Chorna NE, Neary JT, Sun GY, Gonzalez FA, Seye CI, Erb L: Molecular determinants of P2Y₂ nucleotide receptor function: implications for proliferative and inflammatory pathways in astrocytes. *Mol Neurobiol* 2005;31:169–183.
- 8 Camden JM, Schrader AM, Camden RE, Gonzalez FA, Erb L, Seye CI, Weisman GA: P2Y₂ nucleotide receptors enhance alpha-secretase-dependent amyloid precursor protein processing. *J Biol Chem* 2005;280:18696–18702.
- 9 Carini R, Alchera E, De Cesaris MG, Splendore R, Piranda D, Baldanzi G, Albano E: Purinergic P2Y₂ receptors promote hepatocyte resistance to hypoxia. *J Hepatol* 2006;45:236–245.
- 10 Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG: ATP release guides neutrophil chemotaxis via P2Y₂ and A3 receptors. *Science* 2006;314:1792–1795.
- 11 Seye CI, Yu N, Jain R, Kong Q, Minor T, Newton J, Erb L, González FA, Weisman GA: The P2Y₂ nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *J Biol Chem* 2003;278:24960–24965.
- 12 Mediero A, Peral A, Pintor J: Dual roles of diadenosine polyphosphates in corneal epithelial cell migration. *Invest Ophthalmol Vis Sci* 2006;47:4500–4506.
- 13 Greig AV, Linge C, Cambrey A, Burnstock G: Purinergic receptors are part of a signaling system for keratinocyte proliferation, differentiation, and apoptosis in human fetal epidermis. *J Invest Dermatol* 2003;121:1145–1149.
- 14 Ahn JS, Camden JM, Schrader AM, Redman RS, Turner JT: Reversible regulation of P2Y₂ nucleotide receptor expression in the duct-ligated rat submandibular gland. *Am J Physiol Cell Physiol* 2000;279:C286–C294.
- 15 Schrader AM, Camden JM, Weisman GA: P2Y₂ nucleotide receptor up-regulation in submandibular gland cells from the NOD. B10 mouse model of Sjogren's syndrome. *Arch Oral Biol* 2005;50:533–540.
- 16 Seye CI, Gadeau AP, Daret D, Dupuch F, Alzieu P, Capron L, Desgranges C: Overexpression of P2Y₂ purinoceptor in intimal lesions of the rat aorta. *Arterioscler Thromb Vasc Biol* 1997;17:3602–3610.
- 17 Seye CI, Kong Q, Erb L, Garrad RC, Krugh B, Wang M, Turner JT, Sturek M, González FA, Weisman GA: Functional P2Y₂ nucleotide receptors mediate uridine 5'-triphosphate-induced intimal hyperplasia in collared rabbit carotid arteries. *Circulation* 2002;106:2720–2726.
- 18 Godecke S, Decking UK, Godecke A, Schrader J: Cloning of the rat P2u receptor and its potential role in coronary vasodilation. *Am J Physiol* 1996;270:C570–C577.
- 19 Pearson JD, Gordon JL: P2 purinoceptors in the blood vessel wall. *Biochem Pharmacol* 1989;38:4157–4163.
- 20 Lustig KD, Sportiello MG, Erb L, Weisman GA: A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionized forms of ATP and UTP. *Biochem J* 1992;284:733–739.
- 21 Hoebertz A, Mahendran S, Burnstock G, Arnett TR: ATP and UTP at low concentrations strongly inhibit bone formation by osteoblasts: a novel role for the P2Y₂ receptor in bone remodeling. *J Cell Biochem* 2002;86:413–419.
- 22 Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, Burnstock G, Arnett TR: Extracellular nucleotides block bone mineralization in vitro: evidence for dual inhibitory mechanisms involving both P2Y₂ receptors and pyrophosphate. *Endocrinology* 2007;148:4208–4216.
- 23 Douillet CD, Robinson WP 3rd, Milano PM, Boucher RC, Rich PB: Nucleotides induce IL-6 release from human airway epithelia via P2Y₂ and p38 MAPK-dependent pathways. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L734–L746.
- 24 Geary C, Akinbi H, Korfhagen T, Fabre JE, Boucher R, Rice W: Increased susceptibility of purinergic receptor-deficient mice to lung infection with *Pseudomonas aeruginosa*. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L890–L895.
- 25 Buscher R, Hoerning A, Patel HH, Zhang S, Arthur DB, Grasmann H, Ratjen F, Insel PA: P2Y₂ receptor polymorphisms and haplotypes in cystic fibrosis and their impact on Ca²⁺ influx. *Pharmacogenet Genomics* 2006;16:199–205.
- 26 Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR: Effect of loss of P2Y₂ receptor gene expression on nucleotide regulation of murine epithelial Cl[−] transport. *J Biol Chem* 1999;274:26461–26468.
- 27 Tesson L, Cozzi J, Menoret S, Remy S, Usal C, Fraichard A, Anegón I: Transgenic modifications of the rat genome. *Transgenic Res* 2005;14:531–546.
- 28 Koch AK: Experimental modeling and research methodology; in Suckow MA, Weisbroth SH, Franklin CL (eds): *The Laboratory Rats*, ed 2. San Diego, Academic Press, 2005, pp 587–625.
- 29 Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D: Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002;295:868–872.
- 30 Bryda EC, Pearson M, Agca Y, Bauer BA: Method for detection and identification of multiple chromosomal integration sites in transgenic animals created with lentivirus. *Biotechniques* 2006;41:715–719.
- 31 Yang H, Agca Y, Cheng P, Yang J, Agca C, Chan AWS: Enhanced transgenesis by intracytoplasmic injection of bald lentivirus. *Genesis* 2007;45:177–183.
- 32 Schorpp M, Jager R, Schellander K, Schenkel J, Wagner EF, Weiher H, Angel P: The human ubiquitin C promoter directs high ubiquitous expression of transgenes in mice. *Nucleic Acids Res* 1996;24:1787–1788.
- 33 Armstrong DT, Opavsky MA: Superovulation of immature rats by continuous infusion of follicle-stimulating hormone. *Biol Reprod* 1988;39:511–518.
- 34 Bavister BD, Leibfried ML, Lieberman G: Development of preimplantation embryos of the golden hamster in a defined medium. *Biol Reprod* 1983;28:235–247.
- 35 Pfaff RT, Agca Y, Liu J, Woods EJ, Peter AT, Critser JK: Cryobiology of rat embryos I: determination of zygote membrane permeability coefficients for water and cryoprotectants, their activation energies, and the development of improved cryopreservation methods. *Biol Reprod* 2000;63:1294–1302.
- 36 Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH: Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci USA* 1980;77:7380–7384.
- 37 Mullins JJ, Peters J, Ganten D: Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 1990;344:541–544.
- 38 Agca Y, Critser JK: Assisted reproductive technologies and genetic engineering in rats; in Suckow MA, Weisbroth SH, Franklin CL (eds): *The Laboratory Rats*, ed 2. San Diego, Academic Press, 2005, pp 165–190.
- 39 Charreau B, Tesson L, Soullillou JP, Pourcel C, Anegón I: Transgenesis in rats: technical aspects and models. *Transgenic Res* 1996;5:223–234.
- 40 Mullins LJ, Brooker G, Mullins JJ: Transgenesis in the rat. *Methods Mol Biol* 2002;180:255–270.
- 41 Jaenisch R, Harbers K, Jahner D, Stewart C, Stuhlmann H: DNA methylation, retroviruses, and embryogenesis. *J Cell Biochem* 1982;20:331–336.
- 42 Chan AWS, Homan EJ, Ballou LU, Burns JC, Bremel DR: Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. *Proc Natl Acad Sci USA* 1998;95:14028–14033.
- 43 Pfeifer A, Ikawa M, Dayn Y, Verma IM: Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci USA* 2002;99:2140–2145.

- 44 Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL: Production of transgenic rats by lentiviral transduction of male germ-line stem cells. *Proc Natl Acad Sci USA* 2002;199:14931–14936.
- 45 Popova E, Bader M, Krivokharchenko A: Strain differences in superovulatory response, embryo development and efficiency of transgenic rat production. *Transgenic Res* 2005;14:729–738.
- 46 Masaki Y, Sugai S: Lymphoproliferative disorders in Sjögren's syndrome. *Autoimmun Rev* 2004;3:175–182.
- 47 Johnson S, Hulton SA, Brundler MA, Moss C, Huissoon A, Taylor CM: End-stage renal failure in adolescence with Sjögren's syndrome autoantibodies SSA and SSB. *Pediatr Nephrol* 2007;22:1793–1797.
- 48 Iwanaga N, Kamachi M, Fujikawa K, Aramaki T, Izumi Y, Arima K, Tamai M, Aratake K, Nakamura H, Origuchi T, Ida H, Kawakami A, Taguchi T, Eguchi K: Membranous glomerulonephritis and non-Hodgkin's lymphoma in a patient with primary Sjögren's syndrome. *Intern Med* 2007;46:191–194.
- 49 Hard GC, Khan KN: A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. *Toxicol Pathol* 2004;32:171–180.
- 50 Katsifis GE, Moutsopoulos NM, Wahl SM: T lymphocytes in Sjögren's syndrome: contributors to and regulators of pathophysiology. *Clin Rev Allergy Immunol* 2007;32:252–264.
- 51 Vaudo G, Bocci EB, Shoenfeld Y, Schillaci G, Wu R, Del Papa N, Vitali C, Delle Monache F, Marchesi S, Mannarino E, Gerli R: Precocious intima-media thickening in patients with primary Sjögren's syndrome. *Arthritis Rheum* 2005;52:3890–3897.
- 52 Kellerman D, Evans R, Mathews D, Shaffer C: Inhaled P2Y₂ receptor agonists as a treatment for patients with cystic fibrosis lung disease. *Adv Drug Deliv Rev* 2002;54:1463–1474.
- 53 Schrader AM, Camden JM, Weisman GA: P2Y₂ nucleotide receptor up-regulation in submandibular gland cells from the NOD.B10 mouse model of Sjögren's syndrome. *Arch Oral Biol* 2005;50:533–540.